Bacteriophage Therapy for *Staphylococcus aureus* Biofilm–Infected Wounds: A New Approach to Chronic Wound Care

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Background: Bacterial biofilms, which are critical mediators of chronic wounds, remain difficult to treat with traditional methods. Bacteriophage therapy against biofilm has not been rigorously studied in vivo. The authors evaluate the efficacy of a species-specific bacteriophage against *Staphylococcus aureus* biofilm–infected wounds using a validated, quantitative, rabbit ear model.

Methods: Six-millimeter dermal punch wounds in New Zealand rabbit ears were inoculated with wild-type or mutant, biofilm-deficient *S. aureus*. In vivo biofilm was established and maintained using procedures from our previously published wound biofilm model. Wounds were left untreated, or treated every other day with topical *S. aureus*–specific bacteriophage, sharp débridement, or both. Histologic wound healing and viable bacterial count measurements, and scanning electron microscopy were performed following harvest.

Results: Wild-type *S. aureus* biofilm wounds demonstrated no differences in healing or viable bacteria following bacteriophage application or sharp débridement alone. However, the combination of both treatments significantly improved all measured wound healing parameters ($p < 0.05$) and reduced bacteria counts ($p = 0.03$), which was confirmed by scanning electron microscopy. Bacteriophage treatment of biofilm-deficient *S. aureus* mutant wounds alone also resulted in similar trends for both endpoints ($p < 0.05$).

Conclusions: Bacteriophages can be an effective topical therapy against *S. aureus* biofilm–infected wounds in the setting of a deficient (mutant) or disrupted (débridement) biofilm structure. Combination treatment aimed at disturbing the extracellular biofilm matrix, allowing for increased penetration of species-specific bacteriophages, represents a new and potentially effective approach to chronic wound care. These results establish principles for biofilm therapy that may be applied to several different clinical and surgical problems. (*Plast. Reconstr. Surg.* 131: 225, 2013.)

The impact of bacterial biofilms on the pathogenesis and maintenance of chronic, nonhealing wounds has been established within the scientific literature.[1–10] Defined as a surface-adhered, complex community of aggregated bacteria encased within a self-secreted matrix of extracellular polymeric substance, biofilm bacteria possess a diverse set of virulence, defense, and survival mechanisms that distinguish them from traditionally studied, free-floating, "planktonic" bacteria. These include an inherent, physical protection against host inflammatory cells and antibiotic penetration by its self-secreted extracellular polymeric substance,[11,12] and intricate cell-to-cell signaling pathways that are specific to different

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bacterial species.[3,4,13,14] Although the majority of biofilm research has been conducted in vitro, the development of in vivo model systems to study wound biofilm has expanded the translatability of these findings to the clinical setting.[14–24]

Given the enormous financial and emotional burden associated with chronic wound management,[25–30] continued research aimed at treating biofilm, among other causative factors, is critical. However, with only a limited understanding of the biofilm phenotype to date, the development of effective therapies against wound biofilm remains a complex and challenging endeavor. Previous studies have focused on the development of specific dressings or topical therapies, which have shown only mixed efficacy to date.[31–34] Meanwhile, with a growing knowledge of biofilm signaling pathways in vitro, others have aimed to develop targeted molecular-based therapies that have had only minimal verification to date in vivo.[35–37] Our group has taken a principle-based approach to wound biofilm therapy, demonstrating that traditional therapies such as débridement, lavage, and topical antibiotics can be potentially effective when performed in combination and at an increased frequency.[22] However, given the robust durability and virulence of biofilm in the face of host defenses, a continued effort toward innovative treatment principles and solutions is needed.

There has recently been growing interest in the use of bacteriophages for the treatment of bacterial infections, particularly biofilm.[38–48] Bacteriophages are ubiquitous bacterial viruses that infect and kill bacteria through cell lysis but are otherwise harmless to human cells.[39] Several studies have demonstrated the ability of bacteriophages to treat infectious diseases in plants, animals, and humans, including those caused by multidrug-resistant bacterial strains.[44] However, despite these promising results, the literature surrounding bacteriophage therapy against biofilm remains limited, with the majority of studies using artificial, in vitro systems that are difficult to translate to the in vivo wound biofilm setting.[38–46]

In an effort to better characterize the therapeutic potential of bacteriophages against biofilm, we used our validated, in vivo, rabbit ear biofilm model to evaluate the efficacy of a *Staphylococcus aureus*–specific phage against established *S. aureus* wound biofilm. Building on principles established from our previous work,[22] we demonstrated greater improvements in wound healing and biofilm reduction when bacteriophage therapy was combined with surgical débridement than when either modality was used alone. We further investigated the mechanism behind the synergy of these treatments by using a biofilm-deficient mutant of *S. aureus* which, without an intact, protective extracellular polymeric substance matrix, was effectively treated with topical bacteriophage application. With this work, we hoped to reinforce our established biofilm therapeutic principles and introduce a novel approach to clinical chronic wound care.

MATERIALS AND METHODS

Animals

Under a protocol approved by the Animal Care and Use Committee at Northwestern University, adult New Zealand White rabbits (aged 3 to 6 months and weighing approximately 3 kg) were acclimated to standard housing and fed ad libitum. All animals were housed in individual cages under constant temperature and humidity with a 12-hour light/dark cycle. A total of 25 animals were used for this study.

Bacterial Strains and Culture

Wild-type and biofilm-deficient strains of *S. aureus*, UAMS-1 and UAMS-929, respectively, were used for wound infection. The UAMS-929 mutant is deficient in the accessory regulator protein sarA, which is known to modulate the expression of enzymes responsible for polysaccharide intercellular adhesin formation. As one of the critical mediators of biofilm formation, the lack of polysaccharide intercellular adhesin has been shown to reduce its capacity to form biofilm,[49] with a resultant increased susceptibility to topical antibiotics in vitro[50] and in vivo.[51]

S. aureus was grown overnight at 37°C on *Staphylococcus* Isolation Agar (Hardy Diagnostics, Santa Maria, Calif.) and subcultured in tryptic soy broth at 37°C until log-phase was achieved. Bacteria were harvested and washed in phosphate-buffered saline three times by centrifugation at 5000 rpm for 5 minutes at 20°C. An optical density at the 600-nm wavelength was measured and bacterial solution diluted to match an optical density at the 600-nm wavelength equivalent to 10^5 colony-forming units/μl, which was predetermined empirically.

Wound Protocol and Infection Model

Wounding, bacterial infection, and biofilm formation were adapted from principles established in our previously published in vivo wound biofilm model.[21] Rabbits were anesthetized with intramuscular injection of a ketamine (22.5 mg/kg) and xylazine (3.5 mg/kg) mixture before sur-

gery. Ears were shaved, sterilized with 70% ethanol, and injected intradermally with a solution consisting of 1% lidocaine and 1:100,000 epinephrine at the planned wound sites. Six full-thickness dermal wounds, 6 mm in diameter, were created on the ventral ear down to perichondrium and dressed with Tegaderm (3M Health Care, St. Paul, Minn.), a semiocclusive transparent film. Individual biofilm wounds were inoculated with wild-type or mutant *S. aureus* on postoperative day 3. Bacterial solutions were diluted such that each wound was inoculated with a total of 10^6 colony-forming units of bacteria at a volume of 10 μl. Bacteria were allowed to proliferate in vivo under the Tegaderm dressing. Topical antibiotics (Mupirocin 2% ointment; Teva Pharmaceuticals, Sellersville, Pa.) were applied on postoperative day 4 to eliminate free-floating, planktonic-phase bacteria, leaving a predominately biofilm-phase phenotype. To prevent seroma formation and regrowth of planktonic bacteria, thus maintaining a biofilm-dominant infection, an antimicrobial, absorbent dressing containing polyhexamethylene biguanide (Telfa AMD; Tyco Healthcare Group, Mansfield, Mass.) was applied to biofilm wounds on postoperative days 5 and 6 and then every other day until harvest. All dressings were checked daily throughout the protocol. Multiple iterations with this established model[21] have demonstrated the formation of consistent levels of wound biofilm, with predictable end-effects on our host system.

Study Design and Treatment Protocol

Rabbit wounds infected with wild-type *S. aureus* were designated to one of four experimental study arms: untreated, sharp débridement alone, topical bacteriophage therapy alone, or a combination of débridement and bacteriophage therapies. *S. aureus* mutant–infected wounds underwent bacteriophage therapy alone or were left untreated. Sharp débridement was completed using a no. 15 scalpel (Becton Dickinson AcuteCare, Franklin Lakes, N.J.), removing any purulent exudate and debris from the wound bed until it appeared visibly clean. Bacteriophage treatments were performed using a *S. aureus*–specific bacteriophage (generously provided by MicroPhage, Inc., Longmont, Colo.) with previously demonstrated activity against UAMS-1 in vitro (data not shown). Bacteriophage was applied in an approximately 1:1 ratio to the initially applied concentration of bacteria of 10^6 colony-forming units/μl. All treatments were administered to infected wounds every other day starting on postoperative day 6, the time at which a steady-state, predominantly biofilm infection is present.[21–24] After each treatment, new Telfa and Tegaderm dressings were reapplied. On postoperative day 12, after the animals were euthanized by intracardiac Euthasol (Virbac Animal Health, Fort Worth, Texas) injection, wounds were harvested for various analyses. All wounds were excised using a 10-mm biopsy punch (Acuderm, Inc., Fort Lauderdale, Fla.).

Viable Bacterial Count Measurements

The dorsal sides of wounds used for bacterial counts were removed to eliminate the inclusion of bacteria outside of the infected wound surface. To recover bacteria, *S. aureus*–infected biofilm wound samples were placed in tubes prefilled with homogenizer beads (Roche, Indianapolis, Ind.). One milliliter of phosphate-buffered saline was added to the tube and homogenized for 90 seconds at 5000 rpm in a MagNA Lyser homogenizer (Roche Diagnostics, Indianapolis, Ind.), followed by sonication (Microson Ultrasonic Cell Disrupter; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) for 2 minutes at 6 to 8 W to disrupt any biofilm present. The resulting solutions were serially diluted and plated onto *Staphylococcus* Isolation Agar plates and incubated overnight at 37°C. Colony-forming unit counts were determined by the standard colony counting method.

Histologic Analysis

Wounds excised for histologic analysis were bisected at their largest diameter for hematoxylin and eosin staining. Tissues were fixed in formalin, embedded in paraffin, and cut into 4-μm sections. Paraffin was removed with a xylene wash, followed by a standard hematoxylin and eosin staining protocol to prepare samples for analysis under a light microscope. Slides were examined for quantification of new epithelial and granulation distances and for total epithelial and granulation areas using a digital analysis system (NIS-Elements Basic Research; Nikon Instech Co, Kanagawa, Japan) as described previously.[21–24] Two blinded, independent observers evaluated all histologic sections, and the results of both examiners were averaged.

Scanning Electron Microscopy

To visualize biofilm structure, wound samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.2), washed three times in phosphate-buffered saline, and dehydrated through an ethanol series and hexamethyldisilazane. Samples were mounted by double-sided tape

to specimen stubs, followed by gold-platinum (50:50) ion coating (108 Auto Sputter Coater; TedPella, Inc., Redding, Calif.). Wounds for scanning electron microscopy had their dorsal sides removed before preparation to allow for better mounting for visualization. Samples were visualized using a Carl Zeiss (Jena, Germany) EVO-40 scanning electron microscope operated at the scanning voltage of 10 kV.

Statistical Analysis

Data are presented as mean ± SE and analyzed using the *t* test (two-tailed and paired) to compare untreated and bacteriophage-treated mutant *S. aureus*–infected wounds. One-way analysis of variance was used to compare differences in wound healing and viable bacterial counts between different wild-type *S. aureus* study groups. The level of significance was set at $p < 0.05$. All analyses were performed using GraphPad Prism, Version 4.0b (GraphPad Software, Inc., La Jolla, Calif.).

RESULTS

Bacterial burden in *S. aureus* biofilm–infected wounds was measured for each study group to understand the efficacy of each treatment relative to untreated controls. Mean viable bacteria counts (Fig. 1) following bacteriophage treatment were not significantly different from biofilm wounds not receiving any treatment. Similarly, sharp débridement of biofilm-infected also resulted in a minimal decrease in the quantity of viable biofilm. However, therapy involving a combination of treatments, débridement followed by topical bacteriophage application, decreased the number of viable bacteria present by two-log fold ($p < 0.001$), or an approximately 99 percent reduction in bacterial burden. This decrease was visualized through scanning electron microscopy (Fig. 2), demonstrating a relatively intact biofilm structure with large amounts of *S. aureus* following bacteriophage or débridement therapy alone. However, combination treatment resulted in identifiable bare areas of wound bed with sparse amounts of visible bacteria, correlating with the measured bacterial counts.

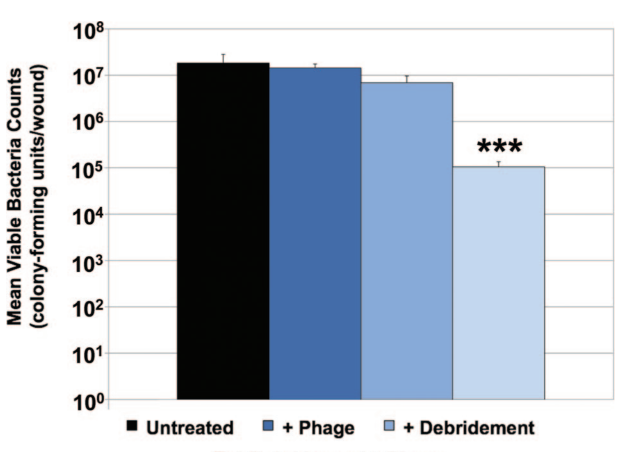

Fig. 1. Mean viable bacterial counts in untreated and treated wild-type *S. aureus* biofilm–infected wounds. Wounds treated with topical bacteriophage or sharp débridement alone demonstrated no difference in bacterial counts when compared with untreated wounds. However, the combination of both therapies resulted in a significant decrease in bacterial burden relative to the other study groups ($n = 10$ to 12 wounds per group; $^{***}p < 0.001$).

Given the known impact of wound biofilms on healing impairment, histologic wound healing measurements were performed following each set of therapies. Photographs of stained histologic sections (Fig. 3) demonstrate distinctly decreased amounts of new epithelial and granulation tissue in single-treatment wounds as compared with dual-therapy wounds, indicating an inability of either treatment alone to improve wound healing relative to untreated controls. These trends were quantified and averaged over several wounds through the measurement of new epithelial and granulation distances and areas (Fig. 4), with the combination of bacteriophage and débridement leading to a significant improvement in all measured histologic parameters relative to both single-treatment groups ($p < 0.05$). These findings indicated a potential synergy between these two modalities in the treatment of wound biofilm, but with an unknown underlying mechanism.

To better understand our findings, additional experiments were performed using the biofilm-deficient, *S. aureus* mutant UAMS-929 within our wound biofilm model. With an inability to form effective biofilm structure, previous work by our group and others has shown that topical antibiotics are effective against this bacterial mutant strain but not against an intact wild-type *S. aureus* biofilm.[51] Similarly, treatment of *S. aureus* mutant–infected wounds with topical bacteriophage alone resulted in a significant reduction in viable bacteria ($p < 0.0001$) (Fig. 5). Corresponding with this decrease in bacterial burden, *S. aureus* mutant wounds treated with bacteriophage demonstrated an improvement in epithelialization and granulation, seen both on histologic section (Fig. 6) and quantitatively across multiple wounds (Fig. 7) ($p < 0.05$). These findings reinforced the theory that bacteriophage can be an effective therapy against wound biofilm in the setting of a disrupted (from

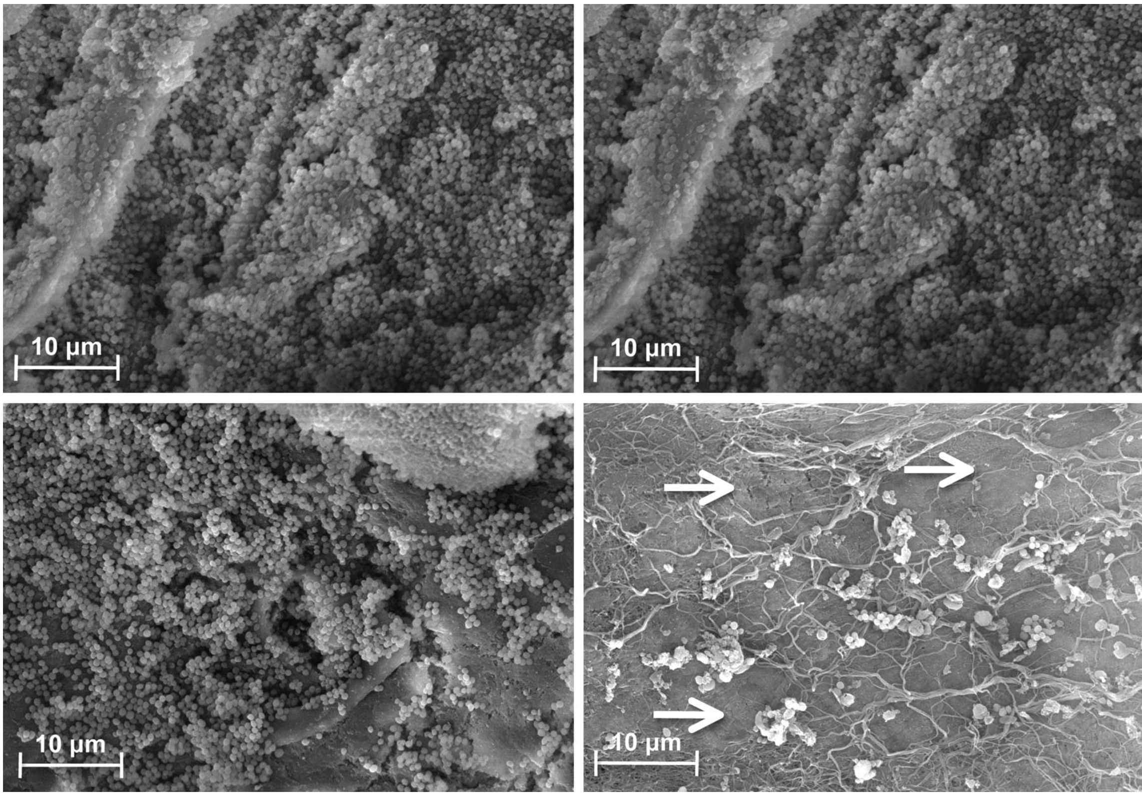

Fig. 2. Scanning electron microscopy of untreated and treated wild-type *S. aureus* biofilm–infected wounds. Corresponding with bacterial counts, single-modality treatment with bacteriophage (*above*, *right*) or sharp débridement (*below*, *left*) alone resulted in minimal differences in wound appearance relative to untreated (*above*, *left*) wounds, including a high density of cocci-shaped *S. aureus*. In contrast, wounds treated with combination therapy resulted in a low density of bacteria with visualized areas of bare wound bed (*arrows*).

débridement) or deficient (because of mutation) extracellular polymeric substance matrix.

DISCUSSION

Chronic wound biofilm continues to be a complex and difficult clinical problem.[1–10,25–30] Despite a steady growth in our understanding of in vivo biofilm pathophysiology within the literature, the development of consistently effective therapeutic regimens has been limited to date.[31–37] To address this need for innovative therapies, we evaluated topical bacteriophage as a novel treatment modality against biofilm, building off of previously described treatment principles[22] and an established in vivo wound biofilm model.[21–24]

Previously published studies have focused on using bacteriophage therapy in the treatment of in vitro biofilms. Fu et al.[38] used an in vitro catheter model to demonstrate a reduction in *Pseudomonas aeruginosa* biofilm formation through pretreatment of the catheter with a specific bacteriophage "cocktail." Similar work using in vitro biofilm culture systems have also shown the efficacy of species-specific phages against *Staphylococcus epidermidis*,[39] *S. aureus*,[43] *P. aeruginosa*,[44] *Escherichia coli*,[45] and *Acinetobacter baumannii*.[46] However, in vitro biofilm systems are unable to incorporate the host defense mechanisms that may develop in the face of an in vivo host inflammatory response, making these findings more difficult to translate to the clinical setting. Alemayehu et al.[47] have shown the in vivo clearance of *P. aeruginosa* biofilm from murine lungs using two different phages. However, to date, this study represents the first in vivo study of phage therapy for wound biofilm. Given our findings, and that phages are specific to bacteria and relatively innocuous to human cells,[39] the incorporation of bacteriophages into clinical wound biofilm therapy represents a particularly attractive idea. However, a review of recent literature by Ryan et al.[48] concluded that the optimization of phage delivery, formulation, and long-term stability are important obstacles to their widespread clinical use, emphasizing the need for continued in vivo research.

Our findings suggest that the combination of sharp débridement and topical bacteriophage

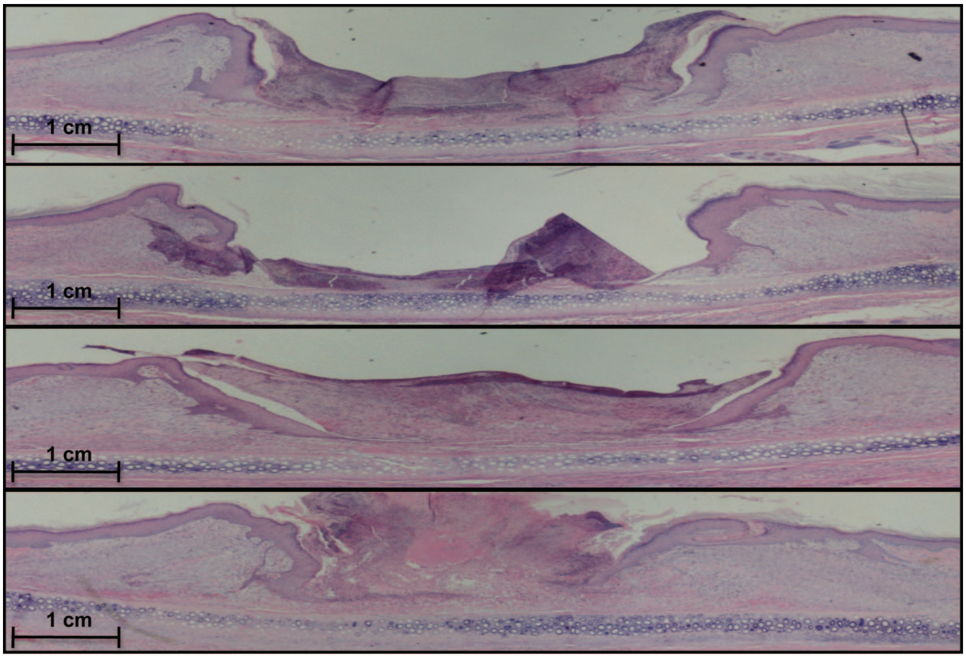

Fig. 3. Comparison of representative histologic sections stained with hematoxylin and eosin between untreated and treated wild-type *S. aureus* biofilm–infected wounds. Wounds treated with débridement followed by topical bacteriophage (*below*) demonstrated the largest amount of epithelial and granulation tissue ingrowth relative to untreated (*above*), débrided (*second row*), and bacteriophage-treated (*third row*) wounds (original magnification, ×20).

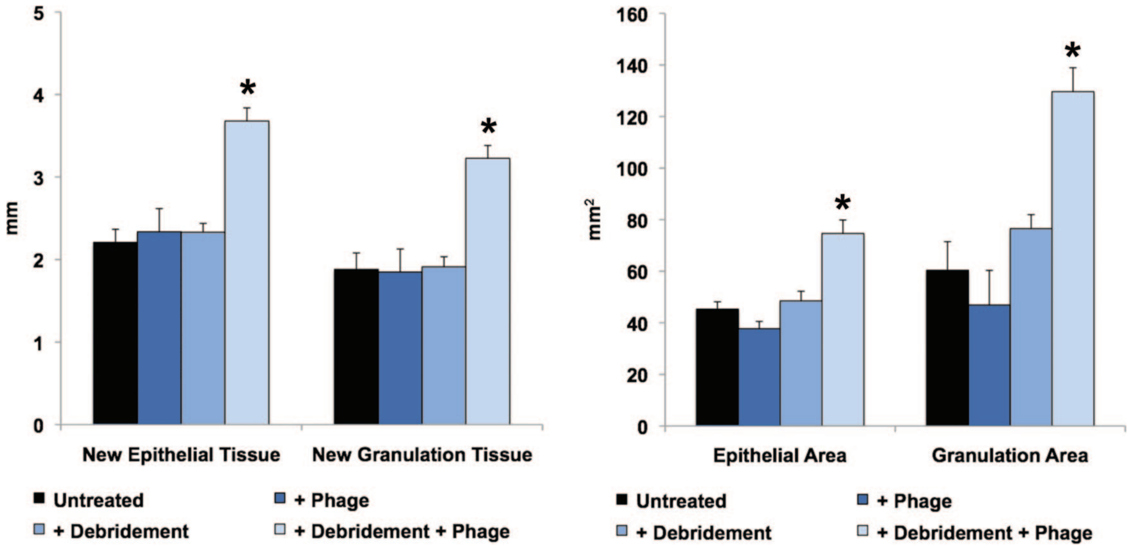

Fig. 4. Comparison of quantitative histologic parameters for untreated and treated wild-type *S. aureus* biofilm–infected wounds. Single-modality treatment wounds (+*Debridement*, +*Phage*) demonstrated amounts of new epithelial and granulation tissue (*left*) and epithelial and granulation areas (*right*) similar to those of untreated wounds. In contrast, combination therapy (+*Debridement,* +*Phage*) resulted in significant improvements in all four measured histologic parameters ($n = 18$ to 20 wounds per group; *$p < 0.05$).

may be an effective treatment against wound biofilm in vivo. However, when treating a bacterial mutant that is deficient in biofilm formation, bacteriophage therapy alone may be successful. Although these findings speak to the need for continued research into phage-based therapies, understanding the underlying principles behind our results may also prove beneficial. In par-

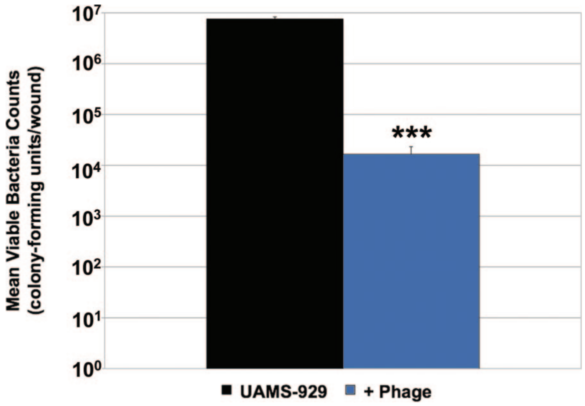

Fig. 5. Viable bacterial counts for *S. aureus* mutant (UAMS-929)–infected wounds with and without bacteriophage treatment alone. Treatment with phage alone resulted in a significant decrease in bacterial counts relative to untreated wounds ($n = 10$ to 12 wounds per group; $^{***}p < 0.001$).

ticular, single-modality therapies with one primary mechanism of action were ineffective against *S. aureus* biofilm, as was seen with *P. aeruginosa* biofilm.[22] Given the durability of biofilm in the face of a harsh external environment, these results emphasize the need for combination, multimodality therapies. As with antibiotics,[11,12] phages may not be capable of penetrating the dense matrix of biofilm extracellular polymeric substance, despite their ability to specifically and effectively lyse bacterial cells. In contrast, mechanical wound care methods, such as sharp débridement or lavage, can provide shearing forces that can disrupt the aforementioned extracellular matrix but may not eliminate the actual bacterial cells. The remaining viable bacteria can subsequently reform

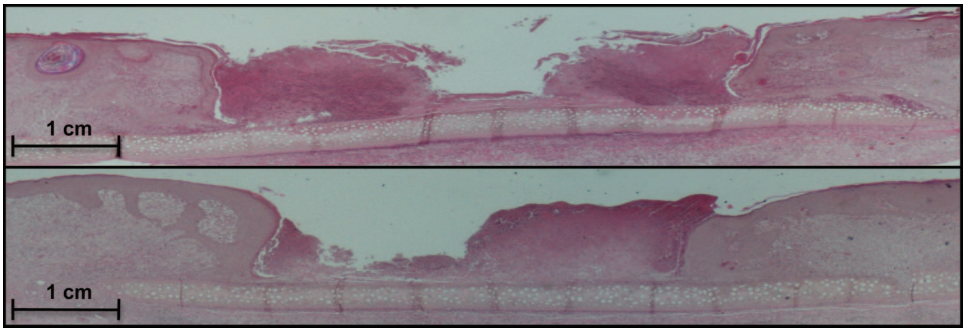

Fig. 6. Representative histologic sections from *S. aureus* mutant–infected wounds with and without bacteriophage treatment. Treated wounds (*below*) revealed larger amounts of epithelial and granulation tissue relative to untreated wounds (*above*) (hematoxylin and eosin; original magnification, ×20).

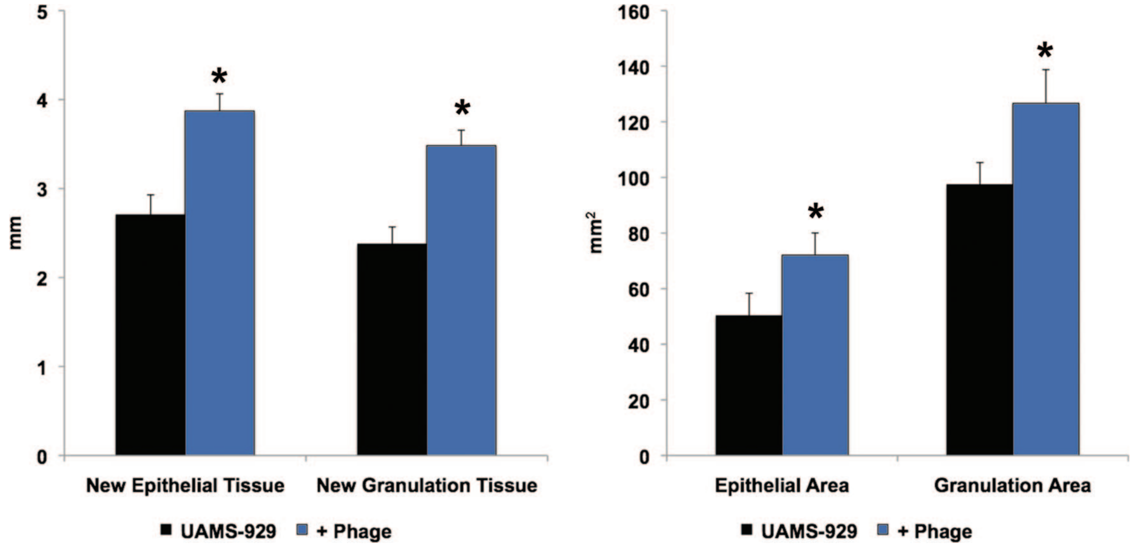

Fig. 7. Quantification of histologic parameters in *S. aureus* mutant (UAMS-929)–infected wounds with and without bacteriophage treatment. Treatment with bacteriophage resulted in significant improvements in epithelial and granulation tissue ingrowth (*left*) and area (*right*) relative to the untreated group, averaged across all wounds ($n =$ 18 to 20 wounds per group; $^{*}p < 0.05$).

a new protective matrix potentially within 24 hours, as previously demonstrated.[21,22] However, the use of a two-prong, combination-based approach of bactericidal [e.g., antibiotics, Silvadene (Monarch Pharmaceuticals, Inc., Bristol, Tenn.), bacteriophages] and mechanical (e.g., débridement, lavage) modalities may represent a simple blueprint for developing future antibiofilm wound care regimens. For example, Ngo et al.[52] recently demonstrated that the combination of topical negative pressure and silver was an effective combination approach against in vitro biofilm over topical negative pressure alone. In our study, the incorporation of phage is particularly advantageous in that it demonstrates comparable efficacy to antibiotics against unprotected, biofilm bacteria, but with less potential for drug resistance.[53]

The efficacy of bacteriophage alone against a biofilm-deficient mutant strain of *S. aureus* emphasizes the importance of the biofilm extracellular polymeric substance to its durability and potentially its virulence. Without a protective extracellular polymeric substance, host defense cells and externally applied therapies can directly interact with bacteria, as in the treatment of traditional, planktonic infections. In particular, this has been shown with the *S. aureus* mutant, UAMS-929, that we used in this study.[49–51] Although not a primary focus of this study, our data also showed that this mutant strain had a decreased impact on wound healing relative to its wild-type counterpart at baseline, with a trend toward increased epithelialization and granulation (Figs. 4 and 7). This would implicate the extracellular polymeric substance as being potentially integral to bacterial virulence as well, a point that we have also recently suggested for *P. aeruginosa*.[24] With a complex structure consisting of polysaccharides, proteins, and nucleotides,[35] the extracellular polymeric substance may act as both a protective barrier and a platform for cell-to-cell signaling and toxin release. Therefore, molecular therapies that specifically target the extracellular polymeric substance matrix (e.g., D-amino acids[35]) may ultimately have a greater impact on biofilm virulence and therefore wound healing than those targeting other, more well-known biofilm signaling pathways.

Despite our novel and rigorous approach, we acknowledge that our study comes with limitations. In particular, we did not extend our analysis to other bacterial species. Although having previously demonstrated similar treatment principles with *P. aeruginosa*,[22] we did not use a *P. aeruginosa*–specific phage or *P. aeruginosa* mutants as part of this study. Future work will be aimed at validating our results with other species and further investigating the potential for phage-based biofilm therapy. Unfortunately, phage therapy itself can also be limiting in that phages are species-specific, thus potentially requiring multiple phages for polybacterial wounds. Also, as with previous studies, the veterinary restrictions associated with frequent animal sedation prevented us from performing multiple treatments on a daily basis. However, we believe that the trends and principles established in this study should continue to hold true with an increased treatment frequency. The translation of this treatment regimen to the clinical setting would allow for the testing of its efficacy with a longer, more frequent treatment timeline.

CONCLUSIONS

The need for innovation in the field of chronic wound care is clear, particularly with regard to treating wound biofilm. An understanding of biofilm pathophysiology, which relies on rigorous molecular and in vitro research, is essential to the development of appropriate wound care principles and novel antibiofilm therapies. However, we also believe that the validation of these innovative treatments using in vivo wound biofilm models is critical for accelerating their transition into the clinical setting. Our in vivo validation of bacteriophage therapy as an adjunctive treatment for wound biofilm establishes a foundation for continued in vivo research and argues for the eventual translation of our experiments into human clinical trials.

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Evidence-Based Medicine: Questions and Answers

Q: Will *PRS* still review, accept, and publish papers with lower levels of evidence?

A: Yes, *PRS* welcomes manuscripts of all Level of Evidence grades and manuscripts that are not amenable to LOE grading. ***The LOE grade should be seen dispassionately as a number, a quantitative indicator of the level of evidence in an article.*** Papers with lower LOE grades (IV and V) are not "worse" than papers with higher LOE grades (I–III); they simply have data of a different level.

It makes sense that randomized, controlled, blinded, multicenter trials with hundreds or thousands of patients and years of follow-up would have a higher level of evidence than a single author's experience in a clinical series. However, given the demands of such studies, it also makes sense that there would be few randomized controlled trials but many single-author series or expert opinions. ***Such series and expert opinions do have value.* PRS *welcomes the submission of such papers and will continue to publish them.***